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The copulatory plug delays ejaculation by rival males and affects sperm competition outcome in house mice

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Abstract

Females of many species mate with multiple males (polyandry), resulting in male-male competition extending to post copulation (sperm competition). Males adapt to the forces arising from postcopulatory sexual selection by altering features of their ejaculate that increase its competitiveness, and/or by decreasing the risk of sperm competition through female manipulation or interference with rival male behaviour. At ejaculation, males of many species deposit copulatory plugs, which are commonly interpreted as a male adaptation to postcopulatory competition, and are thought to reduce or delay female remating. Here, we used a vertebrate model species, the house mouse, to study the consequences of copulatory plug size for postcopulatory competition. We experimentally manipulated plug size after a female's first mating and investigated consequences for rival male behaviour and paternity outcome. We found that even large copulatory plugs were ineffective at preventing female remating, but that plug size influenced rival male copulatory behaviour. Rivals facing larger plugs showed faster paced copulation and longer ejaculation latencies, suggesting that the plug represents a considerable physical barrier. The paternity share of first males increased with a delay of rival male ejaculation, demonstrating a direct fitness benefit for males that produce large copulatory plugs. However, when second males provided little copulatory stimulation, the incidence of pregnancy failure increased, suggesting a benefit of intense and repeated copulation besides plug removal. We discuss potential mechanisms of how plugs influence sperm competition outcome and consequences for male copulatory behaviour.

Key words: Copulatory plug, sperm competition, copulatory behaviour, polyandry, house mouse

Introduction

Females of many species mate with multiple males (polyandry), leading to postcopulatory competition between males (Parker, 1970). Males are predicted to respond to this strong evolutionary force through adaptations in ejaculate production and allocation (Simmons, 2001; Wedell *et al.*, 2002). Males may also maximize their fitness by manipulating or guarding females (Parker, 1970; Gillott, 2003). Copulatory plugs that obstruct the female genital tract and are secreted from males at ejaculation have evolved independently in many different taxa (e.g. insects (Orr and Rutowski 1991) and primates (Dixson and Anderson 2002)), presumably to prevent subsequent inseminations by rival males (Parker, 1970). A role for copulatory plugs in postcopulatory competition has been inferred indirectly in comparative studies on butterflies (Simmons, 2001), spiders (Uhl *et al.*, 2010), rodents (Ramm *et al.*, 2005) and primates (Dixson, 1998). Moreover, positive associations between evolutionary rates of coagulating semen components and indirect measures of sperm competition intensity in rodents (Ramm *et al.*, 2009) and primates (Dorus *et al.*, 2004) further support a role for copulatory plugs in postcopulatory competition. Direct experimental evidence is however mixed. A variety of studies have found an effect of the plug on the outcome of sperm competition (e.g. Masumoto, 1993; Shine *et al.*, 2000; Polak *et al.*, 2001; Kunz *et al.*, 2014), while others have not (e.g. Moreira and Birkhead 2003; Timmermeyer et al. 2010). Given that females benefit from multiple mating in many species (Jennions & Petrie, 2000), they may counteract male attempts to prevent remating (Koprowski, 1992; Stockley, 1997; Friesen *et al.*, 2014), leading to sexual conflict over plug efficacy and co-evolutionary dynamics between both males and females as well as between rival males in plugging and plug removal efficacy (Fromhage, 2012). Thus, even if copulatory plugs are relevant to postcopulatory competition, these evolutionary conflicts over plug efficacy between the sexes and between rivals are likely to lead to situations in which copulatory plugs are not fully effective in preventing female remating.

For rodents, the role of postcopulatory competition in the evolution of copulatory plugs is unclear (Voss, 1979). Comparatively, rodent species with relatively larger testes, a proxy for sperm competition rates, have relatively larger seminal vesicles – the organs responsible for producing plug proteins (Ramm

65 *et al.*, 2005). Within species, a significant effect of the plug on female remating in the guinea pig (Martan
66 & Shepherd, 1976) contrasts with no effect of plug removal in deer mice (Dewsbury, 1988). In house
67 mice, males produce large copulatory plugs from coagulating proteins that are secreted from both the
68 seminal vesicles and the coagulating glands (Gotterer *et al.*, 1955; Rugh, 1968) and that comprise about
69 one third of all semen proteins (Dean *et al.*, 2011). Copulatory plugs may be important for pregnancy
70 initiation by temporally extending vaginal stimulation beyond the ejaculatory reflex (McGill & Coughlin,
71 1970; Leckie *et al.*, 1973). Males that lack the transglutaminase IV gene and cannot form a copulatory
72 plug show reduced fertility, probably because of dramatically reduced sperm transport through the female
73 reproductive tract (Dean, 2013). Thus, aiding sperm transport may be another potential function of the
74 plug. Yet, plugs remain in the female reproductive tract for a prolonged period of time (49% of plugs still
75 present after 24h; Mangels et al. 2015), probably far beyond the length needed for vaginal stimulation and
76 sperm transport. Also, plug removal does not reduce pregnancy rates (Firman & Simmons, 2010), unless
77 removed immediately after ejaculation (Bloch, 1972). Why male mice produce such large and long-lasting
78 plugs might only be understood when considering postcopulatory competition between males. Even if the
79 copulatory plug evolved under selective forces associated with effective sperm transport or pregnancy
80 initiation, it is plausible that the copulatory plug has subsequently evolved to fulfil additional functions
81 related to postcopulatory competition.
82 Multiple paternity is common in natural house mouse populations (Dean *et al.*, 2006; Firman &
83 Simmons, 2008b; Lindholm *et al.*, 2013), and females mate multiply in the lab when given a free choice
84 (Rolland *et al.*, 2003; Manser *et al.*, 2014). Some studies have directly observed female remating after the
85 deposition of a copulatory plug showing that copulatory plugs do not prevent remating (Ramm &
86 Stockley, 2014; Sutter & Lindholm, 2015; Sutter *et al.*, 2015). Nonetheless, large copulatory plugs could
87 be beneficial in the context of sperm competition by delaying ejaculation of rival males to a sub-optimal
88 time relative to ovulation (Parker, 1970; Sutter *et al.*, 2015). In house mice, first males sire the majority of
89 offspring even if plugs are removed (Levine, 1967; Firman & Simmons, 2008a), probably because they
90 ejaculate at an optimal time relative to the release of ova (Gomendio *et al.*, 1998). Preston and Stockley

(2006) showed that males adjust their copulatory behaviour to female oestrus stage, suggesting that males can assess the timing of ovulation. If plugs represent a significant physical barrier to rival males, selection arising from postcopulatory competition is predicted to influence male ability to both deposit efficient plugs in a defensive mating role and to remove plugs in an offensive mating role, possibly involving trade-offs between plug deposition and plug removal skills (Fromhage, 2012).

Sutter et al. (2015) recently showed that repeated ejaculation is accompanied by a decrease in plug size, and used variation in plug size arising from variation in time since a male's last ejaculation to investigate effects on rival male behaviour and paternity share. Larger plugs tended to delay ejaculation by rival males and were associated with a larger first male advantage for paternity share. However, a small sample size due to pregnancy failure and non-independence between plug size and sperm numbers limited the study's conclusions (Sutter *et al.*, 2015). Here, we used a direct experimental approach to assess the role of the copulatory plug in sperm competition in house mice. We used experimental manipulation of plug size in laboratory matings to investigate the effects of plug size variation for rival male mating behaviour. To assess the effects of plug size variation on paternity outcome, we minimised variation in sperm numbers and quality by using sexually rested full brothers of similar intrinsic sperm competitiveness.

Materials and Methods

Experimental animals

Experimental matings were performed using 86 male (aged 2-4 months) and 159 female (aged 2-5 months) laboratory-born F1 to F3 descendants from a free-living population of wild house mice (*Mus musculus domesticus*) in Switzerland (see König and Lindholm 2012). Mice were kept in standard laboratory conditions in a reversed 14L:10D cycle (lights on at 17:30 CET), a temperature of 22-24°C, with food (laboratory animal diet for mice and rats, no. 3430, Kliba) and water provided *ad libitum.* Offspring from monogamous breeding pairs were weaned at 23 days after birth and kept in same sex sibling groups in Macrolon Type III cages (23.5 x 39 x 15 cm). At latest at the onset of aggression

between brothers, males were separated and kept individually in Macrolon Type II cages (18 x 24 x 14 cm). Experimental procedures received ethics approval by the Veterinary Office Kanton Zurich, Switzerland (licence no. 110/2013) and were conducted in accordance with Swiss law.

Plug removal experiment

In controlled laboratory matings we investigated the effect of experimental plug removal. We used virgin females in naturally cycling oestrus and followed a mating protocol described in Sutter & Lindholm (2015). Briefly, a sexually receptive female (based on vaginal cytology; Byers *et al.*, 2012) was introduced into a male's cage after having removed some of the nesting material to facilitate video observation. Every 1-1.5 hours, females were separated from the male and checked for the presence of a copulatory plug, indicating ejaculation by the male (Rugh, 1968). Once a copulatory plug was detected, the female was removed from the male's cage and the plug was either experimentally removed by gently pressing the female against the edge of the handling bin and dislodging the plug with a blunt probe (Firman & Simmons, 2008a), or females were sham treated including the handling but without plug removal. Plugs could often not be removed fully by gentle probing, resulting in partial plug removal in many of the trials. We visually estimated the extent of plug removal and weighed the piece of the plug removed to the nearest 0.1 mg as a proxy for the size of the plug remaining in the female's vagina. The female was then added to the cage of the first male's brother and checked every 30-60 minutes until either a second copulatory plug was observed or until the beginning of the next dark phase. At the end of the experiment, the copulatory plug was again either removed or females were sham treated, matching the treatment after a female's first mating. The female was transferred into a clean cage containing nesting material and *ad libitum* food and water. Experimental trials that did not result in mating were stopped at the end of the dark phase and females were re-tested on a later occasion. Males and females were weighed to the nearest 0.1g before the start of mating trials. We used a paired design with individual males mating in the same order with and without experimental plug removal until we obtained at least one pregnant female from both of the treatments for a given brother pair. Males were sexually rested for a minimum of three days between individual trials to allow sperm and seminal fluid replenishment (Sutter *et al.*, 2015). To account

for potential order effects arising from using initially sexually naïve males, half of the brother pairs commenced in the plug removal treatment and half commenced in the control treatment.

This experiment was part of a series of experiments on reproductive behaviours in relation to the *t* haplotype, a selfish genetic element that shows segregation distortion in males and is frequently found in wild populations (Silver, 1993). A tissue sample taken by earpunch at weaning was used for *t* haplotype genotyping and individual marking. DNA extraction was performed by salt-chloroform extraction (Müllenbach *et al.*, 1989) and *t* genotype was diagnosed by PCR (Schimenti & Hammer, 1990; Lindholm *et al.*, 2013). We have previously shown that males heterozygous for the *t* haplotype (+/*t*) are strongly disadvantaged in postcopulatory competition against wildtype (+/+) males (Sutter & Lindholm, 2015). Here, we predominantly competed full brothers that were equal with respect to *t* genotype (+/+ versus +/+ and +/*t* versus +/*t*) against each other. In some trials however, brothers differed with respect to their *t* genotype. Some of the females involved in these experiments also carried the *t*, but there is no evidence that female genotype influences the outcome of postcopulatory competition (Sutter & Lindholm, 2015). We used full brothers from the same litter to minimise genetic effects on sperm competitiveness other than the *t*. The experimenter was blind with respect to the mice's genotype during mating trials and their analyses.

Validation of plug removal methodology

We ran additional monogamous mating trials to assess plug size variation in natural matings from this population. Mating trials were performed as described above, but females were sacrificed after mating with a single male upon visual detection of a copulatory plug. Plugs were dissected *post mortem* from the female genital tract and weighed to the nearest 0.1 mg. In analogy to the experimental plug removal trials, we visually assessed the extent of plug removal (here based on the amount of plug material remaining attached to the vaginal epithelium). When plugs were completely removed, the proximal part of the plug typically showed a cup form corresponding to the form of the cervix, with a small central protrusion corresponding to the cervical orifice. When plugs broke off, typically only the distal part of the plug could be removed.

169 *Copulatory behaviour*

170 Mating trials were conducted during the dark phase under red light spots. We used video recording with
171 infrared night vision (Sony digital cameras DCR-SR40 and DCR-SR62) to quantify copulatory behaviour
172 and to confirm ejaculation by the second male. Video observation also ensured that the observer was blind
173 with respect to the experimental treatment when quantifying behaviour. Copulatory behaviour of male
174 mice is characterized by initial mounts, a variable number of mounts with intromission (during which the
175 male inserts his penis and performs pelvic thrusts), and ejaculation including the deposition of the
176 copulatory plug (McGill, 1962). One copulatory series includes all mounts and intromissions and ends
177 with ejaculation. We collected detailed behaviour of second-to-mate males on (i) the latency from
178 introduction of the female until the first mount, (ii) the number of copulatory bouts (mounts and
179 intromissions) until ejaculation, (iii) the duration of copulatory bouts, (iv) the latency to ejaculation (from
180 the first mount), and (v) the duration of genital contact during ejaculation. The delay between the two
181 competing males' ejaculations may influence the outcome of sperm competition. Hence, we noted (vi) the
182 timing of ejaculation of both males. Similarly, because males sometimes perform two full copulatory
183 series with the same female and the number of ejaculations influences paternity success (Sutter &
184 Lindholm, 2015; Sutter *et al.*, 2015), we counted (vii) the number of ejaculations of both males. When
185 first males perform a second copulatory series, they may be loosening their own previously deposited
186 plugs. We recorded (viii) the number of post-ejaculatory copulatory bouts performed by the first male to
187 investigate this possibility.

188 *Paternity assignment*

189 Paternity was assigned as described in Sutter and Lindholm (2015). Briefly, we sacrificed females 9 days
190 *post coitum* using gradual CO_2 filling in their home cage and recovered all implanted embryos. We scored
191 12 microsatellites spread across 10 autosomes and performed paternity analysis at a confidence level of
192 95% with a single or no mismatch between offspring and assigned father in CERVUS (Kalinowski *et al.*,
193 2007).

194 *Statistical analyses*

Sample sizes available for statistical analyses are summarised in table 1. Data will be made available on Dryad upon acceptance of the manuscript. Of 100 females used for mating trials, 84 females mated after an average of 1.9 trials (range 1-5). After 42 of these first matings, the copulatory plug was fully or partially removed, resulting in continuous variation in plug removal. Thus, instead of using plug removal as a categorical variable, we used the weight of the plug piece removed as a continuous proxy for the size of the remaining plug to investigate the role of copulatory plugs on male copulatory behaviour and paternity outcome. Using R version 3.1.3 (R Core Team, 2015), we analysed data on the occurrence of remating and pregnancy, on copulatory behaviour and on paternity outcome with either linear (LMM) or generalised mixed models (GLMM), depending on the response variable. In all models, male identity was included as a random factor to avoid pseudoreplication and to account for our paired design. We obtained p-values for fixed effects in LMMs using F-tests, with degrees of freedom based on the Kenward-Roger approximation implemented in the package pbkrtest (Halekoh & Højsgaard, 2014).

Out of 84 mated females, 70 received an ejaculation by their second mate. We tested for an effect of plug removal on remating with a binomial GLMM, using the function glmer in lme4 (Bates *et al.*, 2014). Copulatory behavioural traits were correlated and were reduced using a principal components analysis (PCA). We tested for an effect of plug removal (the size of the plug removed) on the copulatory behaviour of second males with LMMs, using the function lmer in lme4. We fitted full models including either the first or the second principal component of copulatory behaviour of the second male as the dependent variable, and the following variables as fixed effects: the size of the removed plug piece, the number of post-ejaculatory copulatory bouts performed by the first male, the second male's body weight and female body weight. Our full models included 65 mating trials for which we had complete information on all these variables. To avoid biasing effect sizes through removal of non-significant terms (Forstmeier & Schielzeth, 2011), we extracted effect sizes from full models and calculated approximate confidence intervals by multiplying Student's t-values for our sample sizes by standard errors of the predicted values (Crawley, 2007). To improve interpretability, continuous input variables were standardised to a mean of 0 and a standard deviation of 1 as recommended by Schielzeth (2010).

Twelve of the 70 doubly mated females did not become pregnant. A further 15 of the successful trials involved competition between +/*t* and +/+ males, and previous research showed that +/*t* males are strongly disadvantaged in sperm competition (Sutter & Lindholm, 2015). For the final paternity analyses, we thus reduced our dataset to include only sperm competition trials between brothers of the same genotype (i.e. with similar intrinsic sperm competitiveness), since a paternity skew due to the *t* haplotype would have biased effect size estimates for plug removal. We analysed paternity share of the first male (P_1) with binomial GLMMs. The number of embryos sired by the first male was included as the dependent variable and the number of offspring genotyped as the binomial denominator. To investigate how plug removal affects paternity share, we ran a GLMM on P_1, including the weight of the plug piece removed as well as the genotype combination of the brothers and the difference between the number of ejaculations of the first and second male as fixed effects. Male identity was included as a random effect to account for our paired design and input variables were standardised.

Plug removal may affect the outcome of sperm competition indirectly by influencing rival male behaviour as well as directly by physical removal of part of the ejaculate. Thus, we ran a multiple regression analysis on paternity outcome to investigate the relative importance of different explanatory variables. The size of the plug piece removed, the difference in number of ejaculations performed by both males, the difference in body weight between the two males, the interval between the first male's and the second male's first ejaculation (i.e. the duration of exclusive representation of the first male's ejaculate in the female reproductive tract) as well as the *t* genotype of both males were included as fixed effects, and male identity was included as a random effect. Similar to the analyses on copulatory behaviour, we obtained standardised effect sizes, approximate 95% confidence intervals (CI) and p values from the full model. Dispersion parameters of the GLMMs were 1. Figures show untransformed raw data as well as mean model predictions and approximate 95% confidence intervals back-transformed to the original scale and centred for the *t* haplotype input variable.

Results

Experimental plug removal

To test the function of the copulatory plug, we introduced variation in the size of a first male's copulatory plug by either removing part of the plug after ejaculation or leaving the plug intact. In plug removal trials, we removed 28.4 ± 1.9 mg of plug material. We compared the sizes of our experimentally removed plug pieces to plugs completely or partially removed *post mortem* after additional monogamous matings. We included information on the extent of plug removal and on whether the plug had resulted from the male's first or second ejaculation (i.e. four categories: complete removal, majority removal, partial removal, 2nd ejaculation; Fig. 1). There was significant variation between removal categories (LMM: $F_{3,74}$= 30.58, p < 0.001), but experimentally removed plugs did not differ in size from plugs removed *post mortem* ($F_{1,38}$= 0.01, p = 0.904; Fig 1). Thus, weights of completely removed plugs were not significantly different between experimentally and *post mortem* removed plugs (two-sample t-test: 40.6 ± 1.8 mg [mean ± SE] versus 37.7 ± 3.0 mg; t_{15} = 0.88, p = 0.391).

Experimental plug removal affected neither fertility nor fecundity. Pregnancy rates were not significantly different between the plug removal and control groups (30/42 = 71% versus 31/42 = 74%; GLMM: z = 0.25, p = 0.807) and there was no difference in the number of implanted embryos per female in the two treatments (plug removal: 7.7 ± 0.4; control: 8.0 ± 0.3; LMM: $F_{1,32}$= 0.32, p = 0.575).

Copulatory behaviour

We investigated the effect of experimental plug removal on different aspects of copulatory behaviour of second males to mate. Of the 84 females that mated with the first male, 70 mated to ejaculation with the second male. The probability of ejaculation by the second male was not influenced by plug removal (GLMM: 84 trials, 32 brother pairs, z = 1.10, p = 0.271) or by the size of the piece of plug removed (80 trials, z = 0.88, p = 0.379).

The PCA on copulatory behaviour of second males that ejaculated yielded two principal components with eigenvalues larger than one. The first component (PC1) explained 40.9% of the variation in copulatory behaviour and was negatively loaded by the latency to the first mount and the average

copulatory bout duration, and positively by the number of copulatory bouts (Table 2). The second component (PC2) explained 28.5% of the variation and was positively loaded by the latency from the first mount to ejaculation. Higher PC1 scores thus indicated a higher copulatory pace, with an earlier start and more but shorter copulatory bouts. A high PC2 score corresponded to a long ejaculation latency.

Initial analyses showed that the treatment order (i.e. mating experience) of brother pairs did not have an effect on mating behaviour. Likewise, copulatory behaviour of +/*t* males was not different from that of +/+ males (data not shown). Order and genotype were thus dropped from subsequent models. The analysis of PC1 showed that removal of a larger piece of the copulatory plug was associated with slower copulatory pace (smaller PC1 values; standardized effect size *b [95% CI]* = -0.42 [-0.76, -0.08]; $F_{1,50}$= 5.83, p = 0.019; Fig 2a), while neither postejaculatory bouts performed by the first male nor body weight appeared to influence PC1 (Table 3). Similarly, ejaculation latency was shorter (smaller PC2 scores) when a larger plug piece was removed (*b [95% CI]* = -0.42 [-0.68, -0.16]; $F_{1,44}$ = 10.41, p = 0.002; Fig 2b) and shorter when first males had performed more postejaculatory bouts (*b [95% CI]* = -0.24 [-0.56, -0.01] ; $F_{1,56}$= 4.03, p = 0.050; Table 3). Visual examination of individual components of copulatory behaviour suggested that the negative effect of plug removal on PC1 was driven by fewer but longer copulatory bouts (Fig 3).

Paternity share

Out of 84 females that received at least one ejaculation, 23 did not become pregnant. Females that had received an ejaculation by the first and second male were significantly more likely to become pregnant than females that had received no ejaculation by the second male (pregnancy rate remating: 58/70 = 83%; no remating: 3/14 = 21%; GLMM: 84 trials, 32 brother pairs, z = 2.87, p < 0.001), but a GLMM additionally including the number of copulatory bouts performed by the second male suggested that copulatory stimulation was more important for pregnancy than ejaculation *per se* (copulatory bouts: z = 3.04, p = 0.002; ejaculation: z = 1.59, p = 0.113; variance inflation factor = 1.3). The number of implanted

296 embryos was not affected by remating ($F_{1,59}$= 0.02, p = 0.903) or by the number of the second male's
297 copulatory bouts ($F_{1,53}$= 0.43, p = 0.513).

298 Plug removal significantly influenced P_1, alongside the difference in the number of ejaculations of the
299 competing males and their genotype combination. Thus, removal of a larger piece of the first male's
300 copulatory plug reduced his paternity share (Fig 4; GLMM: 40 trials, 24 brother pairs, z = -2.53, p =
301 0.012, *b [95% CI]* = -0.61 [-1.10, -0.12]). To investigate the relative importance of an indirect effect of
302 plug removal via influencing rival male ejaculation timing versus a direct physical effect of plug removal,
303 we performed multiple regression on P_1. The full model showed a positive effect of ejaculation interval on
304 paternity share (Fig 5; GLMM: 39 trials, 24 brother pairs, z = 3.22, p = 0.001, *b [95% CI]* = 1.22 [0.45,
305 2.00]) and a significant effect of the *t* haplotype, with lower P_1 values when two +/*t* brothers competed
306 (Table 3). A direct effect of plug removal on paternity outcome was not supported (z = -0.78, p = 0.434, *b*
307 *[95% CI]* = -0.22 [-0.81, 0.36]; Table 3).

308

309 **Discussion**

310 Copulatory plugs are produced by males in many different animal taxa and are commonly interpreted as
311 an adaptation to sperm competition. However, direct empirical demonstrations of benefits of plugs in a
312 sperm competition context remain scarce. Using experimental variation in copulatory plug size, here we
313 show that copulatory plugs affect rival males' copulatory behaviour and the outcome of sperm
314 competition. The observed effects on copulatory pace and ejaculation latency indicate that copulatory
315 plugs represent a physical barrier to rival males, and that larger plugs are more effective in delaying
316 ejaculation by competitors. Multiple regression analysis on the outcome of sperm competition show that
317 males benefit from large plugs through delaying rival ejaculation: first males that are able to delay their
318 rival's ejaculation for longer secure a larger paternity share than males whose rival's ejaculation is less
319 delayed.

320 *Plug size affects copulatory behaviour*

To investigate the potential of copulatory plugs as mechanical barriers to female remating, we compared experimental trials where we removed plugs (or parts thereof) after a female's first mating to control trials where plugs were left intact. Female remating was not affected by plug removal or plug size. Overall, female remating rate was high (83%), similar to previous laboratory studies on wild-derived house mice (Rolland *et al.*, 2003; Sutter & Lindholm, 2015; Sutter *et al.*, 2015; but see Ramm & Stockley, 2014). When we investigated the effects on copulatory behaviour in more detail, we found significant associations between both major principal components of copulatory behaviour and plug removal variation. Thus, copulatory pace and ejaculation latency decreased when more of the first male's plug was removed: Males facing the obstacle of intact plugs performed more but on average shorter copulatory bouts (Fig 2a, Fig 3) and ejaculated later (Fig 2b). Plug size appeared to affect mainly early mating interactions, with second males performing initially shorter copulatory bouts (left part of Fig 3), probably due to the physical obstacle that intact plugs represented. The decrease in ejaculation latency with the number of first males' postejaculatory bouts further suggests that these bouts contributed to loosening of the plug. Our findings are in line with recent findings from house mice that smaller plugs, caused by short male sexual rest, tended to correlate with males performing fewer copulatory bouts and ejaculating sooner (Sutter *et al.*, 2015). Collectively, available evidence suggests that while copulatory plugs do not prevent female remating, plugs represent physical obstacles that rival males have to remove before they can effectively deposit their own ejaculate, and that larger plugs are more effective at delaying ejaculation by a rival.

The limitations of our experimental approach call for some caution when interpreting the observed effects. First, experimental difficulties with plug removal prevented us from removing the entire plug in many of the trials, limiting the difference in plug size between the two experimental groups and forcing us to use the weight of the removed part of the plug as a proxy for the size of the plug remaining inside the female's vagina. Second, experimental plug removal could have been subject to size-dependent effects. Recently, Mangels *et al.* (2015) showed that after monogamous matings, small plugs persisted in the female reproductive tract for longer than large plugs, suggesting that smaller plugs may be better at

resisting proteolytic degredation by females. If larger plugs were easier to experimentally remove and the remainders of large plugs resisted rival male removal less than remainders of small plugs, our observed association between the size of the plug piece removed and rival male behaviour could have been driven by underlying size-associated differences in plug adherence. However, our additional *post mortem* plug removals from monogamous females allowed us to validate our approach. For these plugs, we were able to assess the extent of plug removal based on plug material adhering to the vaginal epithelium, thus confirming that the weight of the plug piece removed roughly predicted the amount of remaining plug material (Fig 1). We also found that plugs removed after two ejaculations were much lighter than plugs removed after a single ejaculation, confirming a previous finding that plugs produced after repeated ejaculation are smaller than plugs produced after full sexual rest (Sutter *et al.*, 2015). This further qualitatively supports the validity of our approach, as plug removal was performed blind with respect to the number of ejaculations performed by the male (based on video observations).

Larger plugs increase P_1

Our paternity data showed that experimental plug removal affected the outcome of sperm competition. When a larger piece of the first male's plug was experimentally removed, his paternity share decreased. Recently, Sutter *et al.* (2015) showed that a first male's sexual rest (time since last ejaculation) affected sperm competition outcome in house mice, but the experimental design did not separate plug size from ejaculate size. Here, we used fully rested males and removed parts of their copulatory plugs, thus introducing variation in plug size while minimising variation in sperm numbers. Also, we removed plugs after both matings of a female, thus controlling for potential direct effects of plug removal on sperm numbers in the female reproductive tract. Furthermore, for paternity outcome we included only sperm competition trials between full brothers from the same litter. Using males with similar intrinsic sperm competitive abilities and detailed observation of copulatory behaviour enabled us to focus on the effect of ejaculation timing on competitive fertilisation success. Timing effects on paternity share have been demonstrated in hamsters, ground squirrels and rats (Huck *et al.*, 1989; Schwagmeyer & Foltz, 1990; Coria-Avila *et al.*, 2004) with a longer delay of the second male's ejaculation leading to a greater paternity

share for first males. Here, we confirm that the interval between the first male's and the second male's ejaculation is an important determinant of competitive fertilisation success in house mice. Vaginal stimulation immediately after plug deposition has been shown to strongly reduce an ejaculate's fertilisation potential in mice (Bloch, 1972), hamsters (Huck *et al.*, 1989), and rats (Adler & Zoloth, 1970; Coria-Avila *et al.*, 2004). In our experiment, neither plug removal nor copulation with a second male followed the first male's ejaculation immediately. If females are exposed to males in immediate succession, large copulatory plugs may prevent or reduce the likelihood of immediate vaginal stimulation after plug deposition, thus protecting a male's ejaculatory investment from rival males.

Besides timing, the number of ejaculations influences paternity in mice (Sutter & Lindholm, 2015; Sutter *et al.*, 2015) and more generally in rodents (Stockley & Preston, 2004). Our analyses showed that when accounting for the ejaculation interval, the number of ejaculations only showed a positive trend with P_1 success (Table 3). However, as a consequence of our experimental design, there was some collinearity between ejaculation interval and the number of ejaculations performed by the first male. Females were left with their first mate for longer, when he ejaculated twice and his first ejaculation was not detected during a cage check. This collinearity limited our ability to disentangle the relative importance of ejaculation number and ejaculation interval.

Evolutionary implications

We identified fitness-relevant effects of copulatory plugs on house mouse sperm competition that may help explain the evolution and persistence of large copulatory plugs. Larger plugs benefited first males by delaying rival male ejaculation, resulting in a larger paternity share. Given that the response in copulatory behaviour to plug size was mainly seen during the first third of copulatory bouts (Fig 3), it is somewhat surprising that second males did not ejaculate sooner, given the negative effect of ejaculatory delay on their paternity share (Fig 5). However, copulatory stimulation may also increase sperm numbers within an ejaculate (Toner & Adler, 1986), thus affecting its competitiveness. Here, we found that females with more copulatory interactions with second males were more likely to become pregnant, making pregnancy initiation a potential additional incentive for males to maintain a high number of copulations and

Kommentar [AL1]: maybe consider rephrasing to continue your terminology – immediately after deposition is a latency close to zero

Kommentar [AS2]: I don't really see how this would make things more clear. It's a kind of latency that's not really covered with my terminology. It's the latency between the first male's ejaculation and the second male's intromission. I use latency for time between introduction of the female to first mount and ejaculation latency. I think introducing a third kind of latency here is more confusing…
In my dataset, this corresponds to the timing of the first male's ejaculation, which is not really mentioned on it's own, but is included in the interval (time between first and 2nd males' ejaculations).

399 potentially for females to mate with more than one male. The ejaculation latency observed may reflect a
400 male trade-off between increasing copulatory stimulation and reducing ejaculatory delay. An alternative
401 explanation for the negative association between copulatory stimulation and pregnancy failure may be
402 male coercion. Females may have attempted to discriminate against certain second mates by avoiding
403 copulation. The laboratory setting of our mating trials prevented females from escaping, thus potentially
404 allowing males to enforce copulation and ejaculation. Females may then have resorted to discriminating
405 against these males by not initiating or aborting pregnancy.

406 In our laboratory setup, second males were separated from females typically after a single ejaculation.
407 If a longer ejaculation latency induced by a larger plug increases the chance of aggressive takeover by
408 other males or reduces the length of the remaining period of female sexual receptivity available to perform
409 a second ejaculation, the importance of plug size for competitive paternity success may be even more
410 pronounced in a natural setting. Preston and Stockley (2006) found that males were less likely to ejaculate
411 twice if they had provided more copulatory stimulation to females during their first ejaculatory series,
412 providing support for a reduced likelihood of ejaculating twice when ejaculation latency is long. In our
413 setup, males were also fully sexually rested and thus able to produce large plugs. With repeated
414 ejaculation, males become limited in sperm and in seminal fluids required for the copulatory plug. Similar
415 to sperm limitation, seminal fluid limitation may lead to a reduction in paternity skew, when mating with a
416 larger number of females leads to a decrease in postcopulatory competitiveness in each mating event
417 (Preston *et al.*, 2001).

418 Many accounts of copulatory plugs have regarded them as adaptations to sperm competition, but the
419 focus is often put on their potential to prevent remating (Fromhage, 2012). Given the differences in the
420 evolutionary interests of the different actors involved in determining plug deposition and removal
421 efficacy, copulatory plugs are unlikely to end up in a situation where they are completely ineffective or
422 effective. Instead, the interplay between rival males and females will commonly lead to intermediate plug
423 effectiveness and to evolutionarily dynamic changes. Here, we show that copulatory plugs that are
424 ineffective at preventing female remating can still benefit their producers in a sperm competition context

through subtle changes in rival male behaviour (Parker, 1970). Our results contribute to our understanding of the complex dynamics of copulatory plugs in house mice (Mangels *et al.*, 2015), and highlight the importance for investigating fitness consequences of male traits at different stages of reproductive competition.

Concluding remarks

By manipulating copulatory plug size and introducing continuous variation, we show that larger plugs represent a barrier to subsequent rival males, delaying their ejaculation. A delay in rival male ejaculation resulted in a larger paternity share for plug producers, conveying a fitness benefit of depositing large plugs. This may result in strong directional selection for larger plugs and for larger plug-producing accessory glands in the presence of sperm competition.

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Figure legends:

Figure 1: Experimental plug removal. Weights of the plug pieces removed in the main experiment (red) and in additional matings with *post mortem* plug removal (dark red). Raw data are shown alongside with means and standard errors for different categories according to difficulties during plug removal (see main text). Within categories, removal in the main experiment reflected *post mortem* removal, suggesting that the size of the removed plug piece can be used as a proxy for the size of the remaining plug.

Figure 2: The effect of copulatory plug removal on rival male behaviour. a) Copulatory pace (PC1) and b) ejaculation latency (PC2) of the second male to mate are shown as a function of the size of the piece of the first male's plug that was experimentally removed. Red lines and shaded areas represent mean and approximate 95% confidence interval estimates for the effect of experimental plug removal from full models (LMMs), with additional covariates centred to illustrate the effect of plug removal.

Figure 3: Copulatory behaviour in trials with copulatory plug removal (red circles) and control trials (grey diamonds). The mean duration +/- SE of copulatory bouts are shown along their chronological sequence (small light grey and red symbols and error bars). Heights of the bars at the bottom of the figure indicate sample sizes. The large grey and red symbols and error bars represent means +/- SE for total number of copulatory bouts (X axis) and duration (Y axis). The number of copulatory bouts decreased as a function of plug removal, while mean bout duration increased with plug removal (see main text).

Figure 4: The effect of copulatory plug removal on P_1. Paternity share of the first male to mate (P_1) is shown as a function of the size of the piece of the first male's plug that was experimentally

603 removed. Trials with plug removal are represented by red circles and control trials by grey
604 diamonds. The red line and shaded area represent mean and approximate 95% confidence interval
605 estimates for the effect of experimental plug removal from a GLMM including plug removal,
606 centred for ejaculation numbers of the two competing males (large symbols represent two
607 ejaculations by the first male) and for their *t* genotype.

608 Figure 5: The effect of ejaculation delay on P1. Paternity share of the first male to mate (P1) is
609 shown as a function of the interval between the first male's and the second male's first
610 ejaculation (top panel). Trials with plug removal are represented by red circles and control trials
611 by grey diamonds. The blue line and shaded area represent mean and approximate 95%
612 confidence interval estimates for the effect of ejaculation interval from a GLMM. The effect is
613 shown for when there was no difference in ejaculation numbers, and centred for plug removal,
614 body weight difference and the competing males' genotypes. For illustrative purposes, large
615 symbols represent two ejaculations by the first male. Dashed lines show medians of ejaculation
616 intervals for trials with and without experimental plug removal (bottom panel). The blue arrow
617 highlights the decrease in the ejaculation interval that is associated with plug removal, and the
618 corresponding reduction in P_1.

619

620 **Table 1:** Overview of sample sizes for different hierarchical levels of the experimental plug removal experiment and the additional matings. The number of

621 individual females is indicated, with the number of individual males or embryos in brackets.

	Plug removal experiment		**Additional**	**Total**
	Removed	**Control**	***Post mortem***	
Females paired with male (N males)	100 (64)		59 (22)	159 (86)
Females mated (N males)	42 (32)	42 (31)	43 (20)	127 (83)
Females remated (N males)	37 (32)	33 (28)	–	70 (60)
Pregnant females (N paternity/N embryos)	30 (220/232)	28 (213/224)	–	58 (433/456)

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Table 2: Recorded copulatory behavioural traits, their variability indices and results from a principal component analysis (PCA). Eigenvectors in bold were interpreted as contributing significantly to the PC.

Behavioural trait	***Mean***	***SD***	***PC1***	***PC2***
Time of first mount (mount latency) [s]	685	537	**-0.700**	0.400
Number of copulatory bouts	38.3	19.6	**0.869**	0.135
Average duration of copulatory bouts [s]	9.3	4.5	**-0.730**	-0.503
Latency to ejaculation [s]	3591	1909	0.021	**0.897**
In copula duration at ejaculation [s]	12.2	5.0	-0.516	0.437
Eigenvalue	-	-	2.04	1.43
% explained	-	-	40.9%	28.5%

628 **Table 3:** Model summaries for full models on copulatory behavior and sperm competition outcome. Degrees of freedom for F values were based on the

629 Kenward-Roger approximation (Halekoh & Højsgaard, 2014). LMM = linear mixed model, GLMM = generalised linear mixed model.

Model	Response variable	Random effect	Fixed effects	Mean	SD	Fixed effect standardised?	Estimate [approx. 95% CI]	F value/ *z* value	p
LMM	PC1: Copulatory pace	Male ID	Intercept				-0.06 [-0.40, 0.27]	–	–
			Plug piece removed [mg]	15.0	18.1	y	-0.42 [-0.76, -0.08]	5.83	0.019
			Post-ejaculatory bouts	2.5	9.5	y	0.20 [-0.14, 0.55]	1.29	0.261
			Male body weight [g]	26.7	2.3	y	0.27 [-0.08, 0.61]	2.31	0.137
			Female body weight [g]	20.8	1.7	y	0.20 [-0.15, 0.54]	1.19	0.280
LMM	PC2: Ejaculation latency	Male ID	Intercept				-0.02 [-0.34, 0.29]	–	–
			Plug piece removed [mg]	15.0	18.1	y	-0.42 [-0.68, -0.16]	10.41	0.002
			Post-ejaculatory bouts	2.5	9.5	y	-0.28 [-0.56, -0.01]	4.03	0.050
			Male body weight [g]	26.7	2.3	y	0.27 [-0.03, 0.58]	3.04	0.089
			Female body weight [g]	20.8	1.7	y	-0.23 [-0.52, 0.06]	2.32	0.133
GLMM	P1: Paternity share 1st male	Male ID	Intercept				-0.08 [-0.85, 0.68]	-0.22	0.825
			Plug piece removed [mg]	15.1	19.1	y	-0.22 [-0.81, 0.36]	-0.78	0.434
			Ejaculation interval [h]	2.0	0.9	y	1.22 [0.45, 2.00]	3.22	0.001
			Ejaculation number difference	–	–	N	0.64 [-0.09, 1.37]	1.78	0.075
			Body weight difference [g]	-1.3	2.3	y	-0.06 [-0.79, 0.66]	-0.18	0.860
			Male genotype combination	–	–	n	-1.95 [-2.85, -1.04]	-4.36	< 0.001

630